fusion occurs at one cell membrane. Further, recent studies on glucose-mediated insulin release[21] and the effects of CO_2 on vasopressin-induced water permeation[22] suggest that cell acidification may be a more widespread stimulus for exocytosis than previously thought.

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The synthesis and *in vivo* assembly of functional antibodies in yeast

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The yeast *Saccharomyces cerevisiae* can synthesize, process and secrete higher eukaryotic proteins[1-5]. We have investigated the expression of immunoglobulin chains in yeast and demonstrate here (1) the synthesis, processing and secretion of light and heavy chains, (2) the glycosylation of heavy chain, (3) the intracellular localization of these foreign proteins by immunofluorescence, and (4) the detection of functional antibodies in cells co-expressing both chains. This may provide the basis of a microbial fermentation process for the production of monoclonal antibodies. The co-expression of light and heavy chains in *Escherichia coli* has been reported but functional antibodies were not assembled *in vivo*[6,7]. Furthermore, only low-level assembly of these chains was found *in vitro*.

The immunoglobulin λ and μ complementary DNAs used here were isolated from the mouse hybridomas S43 and B1-8, respectively[8,9]. Both hybridomas were raised against the hapten 4-hydroxy-3-nitrophenyl acetyl (NP). The λ and μ cDNAs were placed under the control of the yeast 3-phosphoglycerate kinase (PGK) promoter, on pMA91 bearing the *LEU2* selectable marker[10] (Fig. 1), to form pYλ for the expression of pre-λ, and pYμ for the expression of pre-μ. For co-expression of λ and μ on different plasmids in the same cell, another selectable marker was needed. pLG89 contains two selectable markers, *URA3* and *hph* (ref. 11), therefore the λ coding sequence and PGK promoter from pYλ were excised and inserted into pLG89, to produce pLGλ.

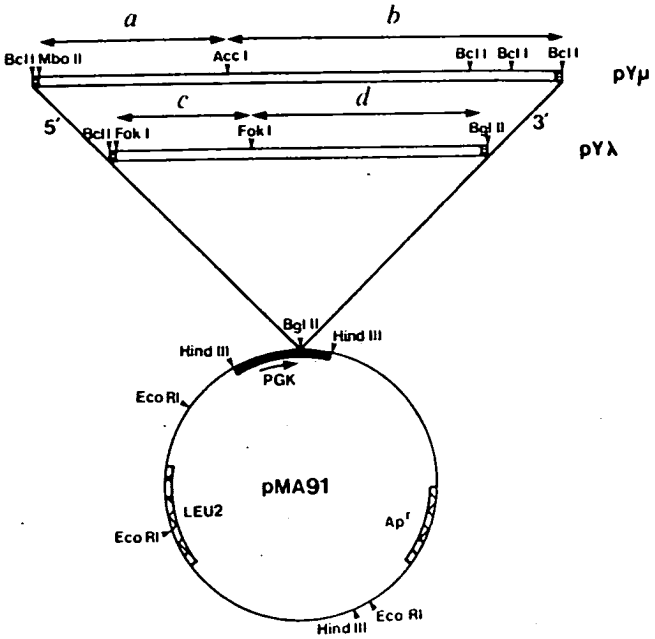

Fig. 1 Plasmids for the expression of immunoglobulin λ and μ chains in yeast. The figure shows the μ and λ inserts of pMA91 which were used to form pYμ and pYλ respectively. Solid bar, PGK sequences; the *LEU2* and Ap^r marker genes are cross-hatched. Sequences derived from synthetic oligodeoxyribonucleotides are indicated by horizontal bars. An arrow indicates the direction of PGK transcription.

Methods. All DNA manipulations were carried out as described elsewhere[22]. For construction of pYλ, the *Hin*dIII fragment of pATλ1-15 (ref. 6) bearing the λ cDNA was isolated, and cut with *Fok*I; 307-base pair (bp) *Fok*I and 512-bp *Fok*I-*Hin*dIII fragments were isolated (fragments *c* and *d*, respectively). Both oligodeoxyribonucleotides[23]: R162 (5'GATCAATGGCCTG-GATT 3') and R163 (5'GTGAAATCCAGGCCATT 3') and pCT54 (ref. 24) cut with *Bcl*I and *Hin*dIII, to form pCTYλ. R162/R163 recreate the 5' coding sequence, and place a *Bcl*I site immediately upstream of the initiation ATG. pCTYλ was digested with *Hin*dIII, blunted with S_1 nuclease and filled-in with T4 DNA polymerase (P-L Biochemicals); the product was ligated with *Bgl*II linkers (5'AGAGATCTCT 3'), then digested with *Bcl*I and *Bgl*II. The fragment bearing the λcDNA was isolated and ligated with *Bgl*II-cut pMA91, to form pYλ. The 5' λ coding sequence of pYλ was isolated and shown to be correct by nucleotide sequencing. The λ coding sequence and PGK promoter were excised from pYλ on a *Hin*dIII fragment, and ligated with *Hin*dIII-cut pLG89 to form pLGλ. For construction of pYμ, the μ cDNA was excised from pCT54μ[25] on a *Hin*dIII fragment, blunted (as for pCTYλ) and ligated with linker R107 (5'TTTTGATCAAAA 3') which contains an internal *Bcl*I site. The ligation product was digested with *Bcl*I and *Acc*I, and the fragment containing the 3' end of μ coding sequence isolated (*b*). Only the *Bcl*I site created by R107 will cut, for internal sites were *dam*-methylated. pCT54μ was cut with *Mbo*II, ligated with R121 (5'GATCAATGGGATGGAGCTGT 3') and R112 (5'CAGCTCCATCCCATT 3') and digested with *Acc*I. The 276-bp *Bcl*I-*Acc*I fragment (fragment *a*) generated was isolated from a 5% polyacrylamide gel. pMA91 cut with *Bgl*II was ligated with both *a* and *b*, to form pYμ. The proximal end of the μ gene was isolated and shown to be correct by nucleotide sequencing[26,27]. Yeast transformations were carried out as described elsewhere[28]. pYλ and pYμ were individually transformed into MD46 (*a*/∝ *pep4.3*/*pep4.3 leu2*/*leu2 arg5.6*/+ +/*trp1* +/*rad52 ade1*/+ *his3*/+; Melanie Dobson, personal communication); pYμ and pLGλ were individually transformed or co-transformed into X4003-5B (*a leu2 ade1 his4 met2 ura3 trp5 gal1*; Yeast Genetic Stock Center, Berkeley, California). Cells containing pYλ or pYμ were selected for by growth in the absence of L-leucine, and pLGλ cells selected for by growth in the absence of uracil.

Cell extracts were subjected to Western blot analysis, using antisera to λ or μ proteins. MD46 cells containing pYλ were found to contain an immunoreactive protein (Fig. 2*a*, lane 3) that co-migrates with authentic B1-8 λ (Fig. 2*a*, lane 1) and with the mature λ protein synthesized by *E. coli* cells containing

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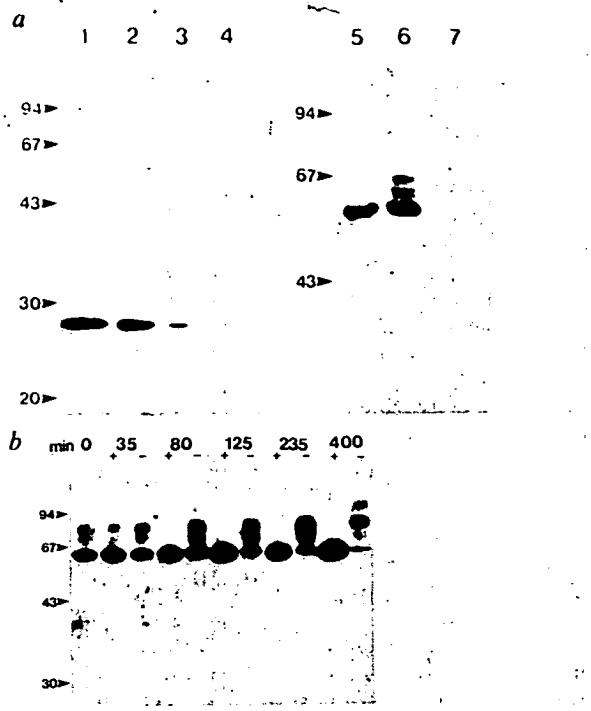

Fig. 2 Analyses of λ and μ proteins in yeast cell extracts. Cells were grown in YMM (2% (w/v) glucose, 0.67% (w/v) Difco yeast nitrogen base without amino acids) to $A_{660} = 1.0$, at 30 °C with shaking, then collected by centrifugation. Cell pellets were disrupted by vortexing with glass beads (40 mesh, BDH) in 50 mM Tris-HCl *p*H 7.6, 1 mM EDTA, and diluted in sample buffer to 1.5% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 5% (v/v) glycerol. Samples were subjected to SDS-polyacrylamide gel electrophoresis[29] and transferred to 0.45-μm nitrocellulose and Western-blotted[30,31] with either rabbit anti-mouse λ antibody (Miles) or rabbit anti-mouse IgM (Bionetics), and challenged with affinity-purified iodinated protein A (2 μCi ml^{-1}; Amersham). Migration of yeast λ and μ was compared with that of B1-8 protein and of mature λ and μ synthesized in *E. coli*[6]. *a*, Lanes 1–4, samples blotted with anti-λ antibody: lane 1, B1-8; lane 2, bacterial λ; lane 3, pYλ/MD46 cell extract; lane 4, MD46 cell extract. Lanes 5–7, samples blotted with anti-IgM: lane 5, bacterial μ; lane 6, pYμ/MD46 cell extract; lane 7, MD46 cell extract. Arrowheads indicate the positions of markers (Pharmacia): phosphorylase *b*, 94 K; bovine serum albumin, 67 K; ovalbumin, 43 K; carbonic anhydrase, 30 K; soybean trypsin inhibitor, 20.1 K. For secretion studies, cells were also grown in YPED (1% (w/v) Difco Bacto yeast extract, 2% (w/v) Difco Bacto peptone, 2% (w/v) glucose) and all cultures were buffered with potassium phosphate *p*H 7.0. In these conditions, we were able to detect the ADH activity in extracts from <0.005 A_{660} units of cells, which would represent lysis of <0.5% of a culture at $A_{660} = 1.0$. At such concentrations, ADH activity remained stable for up to 3 h at 30 °C. Medium supernatants were assayed for ADH by mixing in a cuvette 1 ml of supernatant with 0.3 ml of 500 mM potassium phosphate *p*H 7.0, 100 μl 30 mM NAD (reduced form; Sigma), 0.3 ml 30 mM acetaldehyde (BDH) and 1.3 ml double-distilled water. The decrease in A_{340} was measured with a Gilford spectrophotometer at 30 °C. *b*, Demonstration of μ glycosylation in yeast. Duplicate 50-ml YMM cultures of MD46 containing pYμ were grown in 250-ml baffled flasks, with shaking at 30 °C to $A_{660} = 1.0$ then split into duplicate 20-ml cultures. To one culture, designated '+', we added tunicamycin[12,32] to a concentration of 15 μg ml^{-1}, in 30 μl of dimethyl sulphoxide (DMSO), and to the other culture, designated '−', we added 30 μl of DMSO alone. Cultures were left shaking at 30 °C, and samples taken at various times from both cultures. Cell extracts were prepared and Western-blotted with rabbit anti-mouse IgM and iodinated protein A. Alternating + and − tunicamycin samples are shown and the times of sampling given above the lanes in min. The 0-min sample was taken immediately before DMSO addition. Cells harbouring pYλ were treated similarly, and no difference was found between cultures with or without tunicamycin, when Western-blotted with anti-λ antiserum (data not shown). This result was expected as λ is a non-glycosylated protein in mammalian cells.

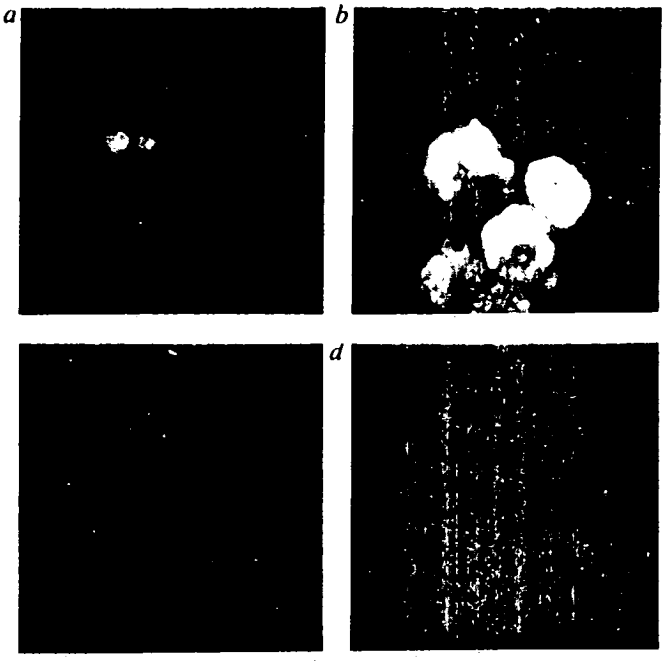

Fig. 3 Intracellular localization of λ and μ protein. The figure shows MD46 cells either untransformed (*c*, *d*), or transformed with pYμ (*a*) or pYMλ (*b*); *a* and *c* were incubated with fluorescein-conjugated anti-μ; *b* and *d* with rhodamine-conjugated anti-λ. ×700.

Methods. Cells were grown in minimal media and converted to spheroplasts with zymolyase 20,000 (50 μg per A_{660} unit in 0.5 ml; Miles), in 1.2 M sorbitol, 50 mM potassium phosphate *p*H 7.0, 15 mM β-mercaptoethanol, 10 mM EDTA, at 30 °C for 30 min. Spheroplasts were washed in 1.2 M sorbitol, and deposited on glass microscope slides using a cytocentrifuge, then fixed in 5% (v/v) acetic acid/95% (v/v) ethanol, at −20 °C overnight. Slides were rehydrated and washed thoroughly in phosphate-buffered saline (PBS), before being stained with 0.1 mg ml^{-1} of fluorescein isothiocyanate-labelled affinity-purified goat anti-mouse IgM or tetramethylrhodamine isothiocyanate-labelled affinity-purified goat anti-mouse λ (both from Southern Biotechnology Associates, Alabama). Slides were washed in PBS, and mounted in glycerol containing 1,4-diazabicylo(2.2.2)octane, before examination by ultraviolet microscopy.

a gene for mature λ (ref. 6) (Fig. 2*a*, lane 2). MD46 cells containing pYμ were found to contain three immunoreactive species (Fig. 2*a*, lane 6). The predominant yeast μ-immunoreactive band had a relative molecular mass (M_r) of ~63,500 (63.5 K) consistent with it being mature, non-glycosylated μ, and co-migrated with the mature μ synthesized by *E. coli* cells containing a gene for mature μ (ref. 6) (Fig. 2*a*, lane 5). In addition, two diffuse bands of greater relative molecular mass were found in MD46 cells containing pYμ (Fig. 2*a*, lane 6). The mature, glycosylated μ of B1-8 has a M_r ~70 K (not shown).

We conclude that the λ immunoreactive species is mature λ, and the ~63.5 K μ species is mature, non-glycosylated μ. On the basis of relative molecular mass, the signal sequences of both proteins have probably been cleaved. The higher-M_r μ bands were shown by two criteria to be glycosylated μ. First, the higher M_r μ was lost from cells treated with tunicamycin, an inhibitor of *N*-linked glycosylation[12]. In cultures without tunicamycin, the amount of higher-M_r μ increased during the time course examined, up to 235-min sample, after which the culture reached stationary phase, and the level of μ decreased (Fig. 2*b*). In cultures containing tunicamycin, the 63.5 K μ species accumulated with this species alone being found after 80 min of tunicamycin treatment. In addition, the higher-M_r form of μ was reduced in size to that of mature, non-glycosylated μ from *E. coli*, when treated with trifluoromethane sulphonic acid, which removes *N*-linked glycosyl groups with high efficiency[13] (data not shown). We conclude that a significant proportion of yeast μ is *N*-glycosylated. However, the yeast

and mammalian μ will probably differ in carbohydrate composition, especially if the yeast μ has outer-chain oligosaccharides[14,15].

Medium supernatants from cultures of MD46 cells transformed with either pYλ or pYμ were found to contain immunoreactive λ or μ protein, respectively. This was shown to be genuinely secreted material, rather than arising from cell lysis, by demonstrating the absence of a cytoplasmic enzyme, alcohol dehydrogenase (ADH), from the supernatants (see Fig. 2 legend). In yeast minimal medium (YMM), up to 10% of λ and 5% of μ in MD46 cultures at A_{660} ~1.0 was found in the medium supernatant, as detected by enzyme-linked immunosorbent assay (ELISA)[25]. In YPED medium (Fig. 2 legend) up to 40% of λ and 15% of μ was found in this fraction. Transformed X4003-5B cells generally yielded higher levels of secreted material. The immunoreactive λ material from the medium supernatants of cultures containing pYλ was shown, by Western blotting, to co-migrate with intracellular λ (data not shown). The secreted μ has not yet been examined.

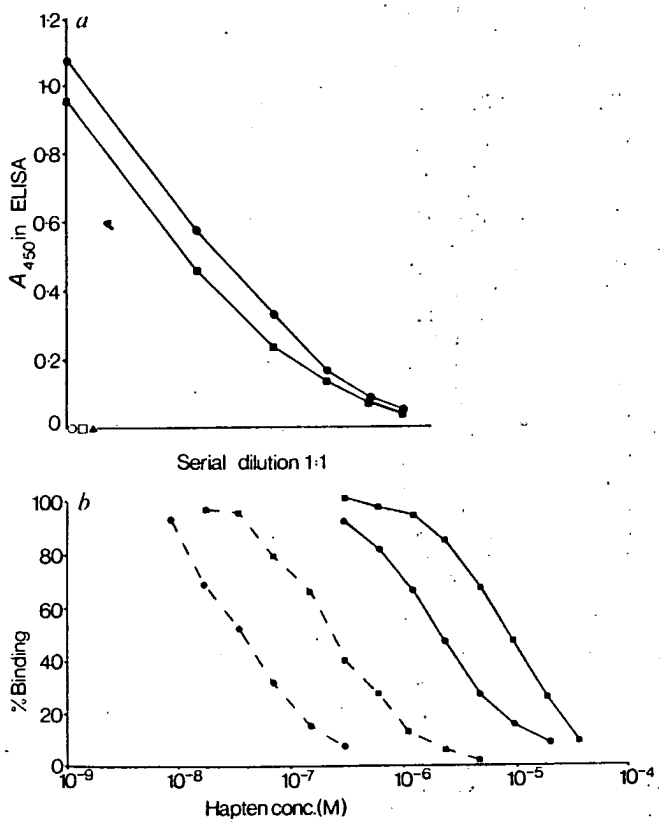

Fig. 4 Analysis of functional antibodies in yeast. Transformed X4003-5B cells were grown in 100-ml volumes of YMM, supplemented with 50 mg l^{-1} of L-tryptophan, L-histidine and L-methionine, and 30 mg l^{-1} of adenine sulphate in 500-ml flasks. Strains not containing pLGλ were supplemented with 30 mg l^{-1} uracil, and those not containing pYμ with 50 mg l^{-1} L-leucine. Cells were collected by centrifugation, then washed and resuspended in 25 mM Tris-HCl *p*H 8.0, 1 mM EDTA, 5% (v/v) glycerol, 0.1% (v/v) Nonidet-P40, 1 mM phenylmethylsulphonylfluoride (extraction buffer), before being lysed by glass bead disruption. 'Insoluble' material was pelleted in a microfuge for 12 min at 4 °C, and the 'soluble' supernatant fraction removed. The soluble fraction was analysed in a two-site sandwich ELISA (NIP assay) which detects μ-chain binding to haptenylated bovine serum albumin (NIP-caproate-BSA)[6]; this assay is sensitive to 60 pg of B1-8 IgM. *a*, Specific hapten binding was examined in the NIP assay of the soluble fraction of pLGλ + pYμ-transformed cells (●, ○), pYμ-transformed cells (▲) and B1-8 (■, □), in the absence (solid symbols) and presence (open symbols) of 30 μM free NIP-caproate. *b*, The heteroclitic nature of yeast antibody activity (●) in soluble fractions from pLGλ + pYμ-transformed cells and B1-8 (■) was examined by comparing binding in the presence of free NIP-caproate (- - -) and NP-caproate (——).

The intracellular locations of λ and μ proteins in yeast were examined by staining fixed yeast spheroplasts with fluorescein or rhodamine-conjugated antisera. We stained both MD46 and X4003-5B strains, harbouring pYμ, pYλ, pLGλ or pYMλ. pYMλ is a pMA91 derivative encoding the mature λ protein, that is, Met-mature λ, not including the signal sequence. Cells containing pYμ (Fig. 3*a*), pYMλ (Fig. 3*b*), pYλ or pLGλ showed a discrete immunofluorescence localized in bodies that appeared on the basis of their morphology to be vacuoles[16,17]. Cells containing pYMλ showed a much greater accumulation of stain than those containing pYλ; this may be explained by the observation that the intracellular concentration of mature λ from pYMλ is up to fourfold greater than that of pre-λ from pYλ, in MD46, by ELISA. Further experiments are required to identify unequivocally the sites of accumulation of immunoreactive λ and μ proteins. If these foreign proteins are being localized in the vacuole, they could be transported there by the secretory pathway[18,19], however, the detection of mature λ from pYMλ in the same structures calls this into question—the cell may be directing these proteins to the vacuole for degradation[20].

Soluble extracts of X4003-5B cells transformed with one or both of pYμ and pLGλ were prepared and analysed by an ELISA that detects binding of B1-8 to solid-phase hapten, in the presence and absence of competing free hapten[6] (Fig. 4*a*); no specific binding was detected for extracts of cells transformed with either pLGλ or pYμ. However, extracts of cells transformed with both plasmids, and expressing both light and heavy chains, showed a strong, specific signal similar to that of the hybridoma B1-8 protein. All detectable solid-phase hapten binding was lost in the presence of 30 μM free hapten. B1-8 is a heteroclitic antibody, and shows greater affinity for the related hapten 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP), than for the hapten NP[21]. The yeast antibody activity also showed that higher free NP than free NIP concentrations were required to inhibit binding of antibody to solid-phase NIP (Fig. 4*b*). In addition, the activities of the yeast antibody and B1-8 showed similar specificity ratios (ratio of concentration of NP to NIP at 50% inhibition) of 37 and 47, respectively. The amount of μ protein in the soluble extract of cells containing pLGλ and pYμ was determined by ELISA, and using B1-8 as a standard, the specific activity of the yeast antibody was found to be ~0.5%. ELISA showed that at A_{660} = 1.0, there was ~500 ng of B1-8 λ equivalent, and 15 ng of B1-8 μ equivalent per ml. No significant amounts of NIP binding activity have been found in concentrated culture media (data not shown).

On the basis of the specific activity of the soluble-fraction yeast antibodies, the efficiency of assembly of functional antibodies is low, although the antibodies show both specific hapten binding and heterocliticity. It will be interesting to characterize further the yeast antibodies in the soluble fraction and to determine whether or not most of the immunoglobulin protein from the insoluble fraction (~75% of total) is also assembled into functional antibodies.

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Functional modifications of cytotoxic T-lymphocyte T200 glycoprotein recognized by monoclonal antibodies

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Plasma membrane glycoproteins of cytotoxic T lymphocytes (CTLs) are involved in the binding to and subsequent destruction of appropriate target cells[1-3]. The electrophoretic profile of surface proteins of mature CTLs, particularly those of high relative molecular mass (M_r), is markedly different from that of naive peripheral T cells or non-cytolytic T cells[4-7], suggesting the possible involvement of these molecules in the activation of CTLs and/or in the lytic process itself. By generating monoclonal antibodies to cell-surface proteins of CTL clones, we have now detected CTL-specific modifications in one of these high-M_r membrane proteins, T200. Although forms of T200 are found on a wide variety of cell types, the neoantigenic determinants recognized by our antibodies are present exclusively on activated T cells and in high concentrations only on CTLs. Furthermore, the expression of the modifications recognized by our antibodies is influenced by soluble factors and also seems to have functional significance, as monoclonal antibodies specific for these novel epitopes block cytolytic activity.

Monoclonal antibodies with specificity for CTL surface recognition structures were produced by immunizing BALB.B mice repeatedly (intraperitoneally) with a C57BL/6-derived CTL clone, B3.3, which is specific for a BALB minor histocompatibility antigen in association with H-2K^b. Spleen cells of immune mice were fused to a myeloma partner, P3-X63 Ag8.653, and the resulting hybridoma supernatants were screened for the ability to block specific target lysis by B3.3. Figure 1*a* depicts the blocking activity of two of these monoclonal antibodies, CT1 and CT2, on clone B3.3 in conditions of saturating antibody. CT1 and CT2 (which are both IgM antibodies) efficiently block specific killing of BALB.B target cells by clone B3.3, CT2 being the more efficient of the two antibodies. In this regard, CT2 was as efficient as the anti-Lyt-2 monoclonal antibody, 53.6.72. Antibody I3/2.3, which is specific for the T200 molecule[8], did not significantly affect specific lysis by this CTL clone.

To determine whether CT1 and CT2 were able to block CTL-mediated killing in conditions not requiring antigen-specific recognition on the part of the CTLs, we tested their ability to inhibit lectin-dependent cell-mediated cytotoxicity (LDCC). Both antibodies were highly efficient as inhibitors of

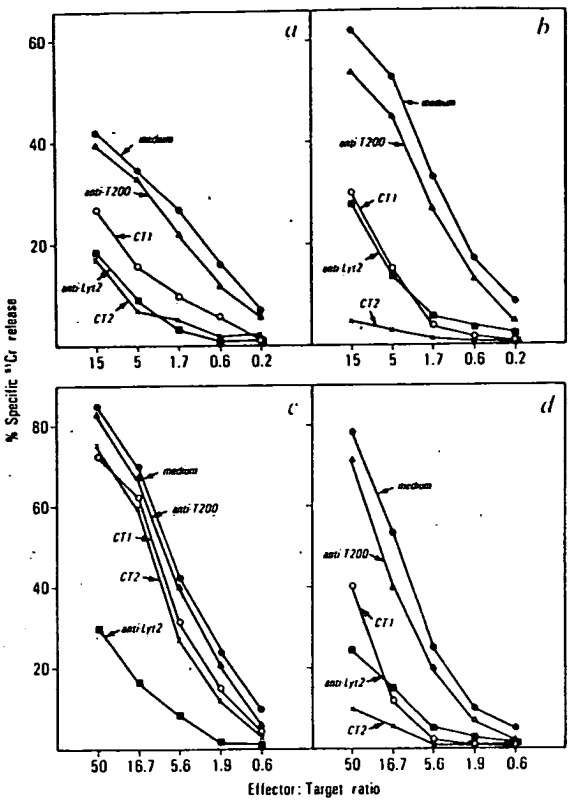

Fig. 1 Inhibition by CT antibodies of cytolysis mediated by cloned CTLs and MLC-derived CTLs. Serial dilutions of effector cells were incubated in 96-well round-bottomed microtitre plates for 30 min at 4 °C with saturating levels of CT1 (○), CT2 (×), I3/2.3 (△, anti-T200, kindly provided by Dr Ian Trowbridge, Salk Institute), 53.6.72 (■, anti-Lyt-2), or medium (●). Effector cells were: *a*, *b*, CTL clone B3.3 (a C57BL/6 clone derived by limiting dilution and specific for a BALB minor histocompatibility antigen in association with H-2K^b); *c*, C57BL/6 spleen cells stimulated for 5 days with irradiated DBA/2 spleen cells (5×10^6 cells of each per well in 24-well plates) in RPMI 1640 culture medium supplemented with 5% fetal calf serum; *d*, MLC cells derived as in *c* but restimulated (5×10^5 responders and 5×10^6 stimulators per well) every 7 days for 4 weeks in medium containing 5% rat Con A supernatant (RCS). ^{51}Cr-labelled target cells (1×10^4) were as follows: *a*, 3-day BALB.B Con A blasts; *b*, P815 H-2^d mastocytoma cells with the addition of 10 μg ml^{-1} PHA; *c*, *d*, P815 cells. After the addition of target cells, the plates were centrifuged for 3 min at 800 r.p.m. to initiate cell contact and incubated at 37 °C for 2 h. Per cent specific lysis was calculated as $100 \times$[(c.p.m. released with effectors) − (c.p.m. released alone)]/[(c.p.m. released by detergent) − (c.p.m. released alone)]. Spontaneous release of P815 target cells was 5% and that of BALB.B Con A blasts 18%. No significant lysis was observed of C57BL/6 Con A blasts (*a*), of P815 cells without the addition of PHA (*b*), or of EL4 cells (*c*, *d*).

phytohaemagglutinin (PHA)-dependent killing of P815 tumour cells by clone B3.3 (Fig. 1*b*). CT1 was comparable with anti-Lyt-2 in blocking LDCC, while the anti-T200 antibody did not inhibit lysis (Fig. 1*b*). (Note that CT1 and CT2 do not bind to the target cells used in these assays; see below.) We also found that five out of five independently isolated CTL clones of various target specificities were blocked by both antibodies. Thus, it was apparent that CT1 and CT2 were not clone-specific or anti-idiotypic in their reactivity. More surprising results were obtained when we examined their effect on heterogenous populations of CTLs generated in mixed lymphocyte culture (MLC). When the effects of these antibodies on the lytic ability of a primary MLC were tested (Fig. 1*c*), only minimal inhibition of specific lysis was observed using CT1, CT2 or the control antibody I3/2.3 (anti-T200), while 53.6.72 (anti-Lyt-2) significantly inhibited lysis. However, quite surprisingly, in inhibition assays using as effectors CTLs from a long-term MLC, CT1 and CT2 produced a significant decrease in specific lysis (Fig. 1*d*). This MLC (and others similarly blocked by CT1 and CT2) were